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Glycerol conversion to 1,3-propanediol by *Clostridium pasteurianum*: cloning and expression of the gene encoding 1,3-propanediol dehydrogenase

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Abstract

When grown on glycerol as sole carbon and energy source, cell extracts of *Clostridium pasteurianum* exhibited activities of glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase and 1,3-propanediol dehydrogenase. The genes encoding the latter two enzymes were cloned by colony hybridization using the *dhaT* gene of *Citrobacter freundii* as a heterologous DNA probe and expressed in *Escherichia coli*. The native molecular mass of 1,3-propanediol dehydrogenase (DhaT) is 440 000 Da. The *dhaT* gene of *C. pasteurianum* was subcloned and its nucleotide sequence (1158 bp) was determined. The deduced gene product (41 776 Da) revealed high similarity to DhaT of *C. freundii* (80.5% identity; 89.8% similarity).

Keywords: *Clostridium pasteurianum*; 1,3-Propanediol dehydrogenase; 1,3-Propanediol; Glycerol fermentation; Type III alcohol dehydrogenase; Glycerol dehydratase

1. Introduction

It has been known for about 60 years that glycerol is fermented by facultatively anaerobic bacteria to 1,3-propanediol, ethanol, 2,3-butanediol, acetic and lactic acids. Of these substances 1,3-propanediol is of industrial interest as a monomer for light-insensitive plastics, and some strains indeed form this diol as the main product. Suitable production organisms belong to the enterobacterial genera *Klebsiella* and *Citrobacter* [1]. Recently, it has been shown that some clostridial species also convert glycerol to 1,3-propanediol [2]. The fermentation pattern is different in that the clostridia form butyric acid as a by-product. Some strains of *Clostridium pasteurianum* produce considerable amounts of butanol and ethanol in addition [3].

The key enzymes and the corresponding genes for glycerol fermentation have been identified and characterized only in *Citrobacter freundii* and *Klebsiella pneumoniae* [4,5]. In the absence of an external oxidant, glycerol is consumed by a dismutation process involving two pathways. Through one pathway glycerol is dehydrogenated by an NAD^+-linked glycerol dehydrogenase (DhaD) to dihydroxyacetone. This product is then phosphorylated by dihydroxyacetone kinase (DhaK) and funnelled to the central metabolism [6]. Through the other pathway glycerol is de-

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hydrated by coenzyme B_{12}-dependent glycerol dehydratase (DhaB, DhaC, DhaE) to form 3-hydroxypropionaldehyde, which is reduced to the major fermentation product 1,3-propanediol by the NADH-linked 1,3-propanediol dehydrogenase (DhaT), thereby regenerating NAD^+ [7,8]. The four key enzymes of this pathway are encoded by the *dha* regulon, the expression of which is induced when dihydroxyacetone or glycerol is present. Recently, we have cloned and expressed the entire *dha* regulon of *C. freundii* in *Escherichia coli* [5]. The genes encoding the four key enzymes and the corresponding gene products have been sequenced and purified [6–8]. In contrast to the 1,3-propanediol-forming enteric bacteria only little is known about the enzymes responsible for glycerol breakdown by clostridia. The activity of glycerol dehydrogenase, glycerol dehydratase and 1,3-propanediol dehydrogenase has been determined in crude extracts of *C. butyricum* [9] and the latter activity in *C. pasteurianum* [2]. To our knowledge, the genes encoding key enzymes involved in glycerol conversion to 1,3-propanediol by clostridia have not been identified and sequenced.

In this report, we describe the cloning and expression in *E. coli* of the genes encoding glycerol dehydratase and 1,3-propanediol dehydrogenase of *C. pasteurianum* and the sequence of the *dhaT* gene.

2. Materials and methods

2.1. Bacterial strains and vectors

C. pasteurianum DSM 525 and *C. freundii* DSM 30040 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *E. coli* ECL707 [4] and DH5α [10] were used as hosts, and the cosmid pWE15 and the plasmid pBluescript SK^+ (Stratagene GmbH, Heidelberg, Germany) were employed as the vectors for cloning experiments.

2.2. Media and growth conditions

C. pasteurianum was grown in a minimal medium according to Kell et al. [11] with 100 mM glycerol as carbon source and *C. freundii* as described previously [5]. *E. coli* was routinely cultivated at 30°C in LB medium [10], which was supplemented with ampicillin (100 μg ml^{-1}) when necessary. Recombinant *E. coli* strains used for expression of the genes involved in glycerol breakdown were grown as described previously [6]. Fermentations were done in Hungate tubes or anaerobic flasks and media were gassed with N_2 for 30 min before sterilization. A modified MacConkey agar (lactose was replaced by 70 mM glycerol) was used to identify glycerol-utilizing recombinant *E. coli* strains.

2.3. Molecular procedures

Chromosomal DNA from *C. pasteurianum* was isolated applying the method of Marmur [12], partially digested with *Eco*RI or *Hin*dIII, and ligated into the above mentioned vectors. Digestion with restriction endonuclease, ligation, packaging of DNA, transduction of cosmids, transformation of plasmids and isolation of recombinant vectors were done according to standard procedures [10]. Transductants were screened on MacConkey-glycerol-ampicillin agar for glycerol utilization, which was indicated by a red color of the colonies.

The subcloning of genes involved in glycerol fermentation of *C. pasteurianum* was performed in heterologous hybridization studies using the *dhaT* gene of *C. freundii* as a probe. As source for the isolation of this gene the recombinant cosmid pRD1 was used, which harbors the entire *dha* regulon of *C. freundii* [5]. Colony hybridization, Southern transfer of DNA fragments to nylon membranes and detection of ^{32}P-labelled probes were done according to Ausubel et al. [10]. DNA sequence was determined by the chain termination method of Sanger et al. [13]. The fidelity of the DNA sequence determined for the insert of pFL2 was confirmed by commercial sequencing (Seqlab, Göttingen, Germany).

2.4. Preparation of cell extracts

Cells of the stationary growth phase from 500 ml anaerobic cultures were harvested by centrifugation at 6000×*g* for 20 min, washed once with 100 mM potassium phosphate buffer (pH 8.0) and resuspended in 2–3 ml of the same buffer. The cells were disrupted by French pressing (1.38×10^8 Pa) and the extract was cleared by centrifugation at

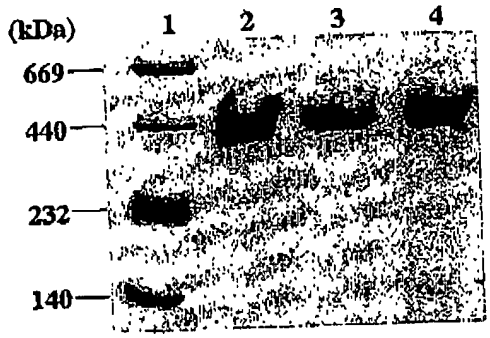


Fig. 1. Nondenaturing polyacrylamide gel electrophoresis and activity staining of 1,3-propanediol dehydrogenase. The crude extracts were subjected to electrophoresis under nondenaturating conditions on polyacrylamide gradient slab gels (4–28%). The protein bands were stained as described in Section 2. Lanes: 1, molecular mass markers; 2, crude extract of *C. freundii*; 3, crude extract of *C. pasteurianum*; 4, crude extract of *E. coli* ECL707/pFL1.

32000×*g* for 35 min at 4°C. All steps were done under anaerobic conditions.

2.5. Enzyme assays

Glycerol dehydrogenase was assayed by the method of Ruch et al. [14] and dihydroxyacetone kinase by the method of Johnson et al. [15]. Glycerol dehydratase was estimated by the 3-methyl-2-benzothiazolinone hydrazone method [16] in 1 min assays with glycerol as substrate. The activity of 1,3-propanediol dehydrogenase was determined as described previously [7]. Protein concentrations were measured by the method of Bradford [17] with bovine serum albumin as standard. All enzyme activities are expressed in μmol min^{-1} mg $protein^{-1}$.

2.6. Determination of molecular mass

Electrophoresis under nondenaturing conditions was carried out on polyacrylamide gradient slab gels (4–28%) at 4°C in Tris-glycine buffer (pH 8.3) by the method of Andersson et al. [18]. Activity staining of 1,3-propanediol dehydrogenase was performed as described by Boenigk [19]. For calculation of the native molecular mass, a commercial high-molecular-mass calibration kit of standard proteins was used.

3. Results and discussion

When grown in minimal medium with 100 mM glycerol as the energy and carbon source in batch culture, *C. pasteurianum* formed 1,3-propanediol, butanol and ethanol as the major fermentation products (data not shown). The four key enzymes, which are known to be responsible for the conversion of glycerol to 1,3-propanediol in enteric bacteria, could be detected. The specific activities determined for glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase and 1,3-propanediol dehydrogenase in cell extracts of *C. pasteurianum* were 4.5, 0.1, 2.2 and 1.7 U mg^{-1}, respectively. These activities were in the same range as in cell extracts of *C. freundii* and *E. coli* ECL707/pRD1, which harbors the entire *dha* regulon of *C. freundii* (Table 1). This result indicated that *C. pasteurianum* ferments glycerol like the 1,3-propanediol-forming enteric bacteria by a dismutation process. This is in accordance with the pathway postulated for the glycerol fermentation of *C. pasteurianum* by Dabrock et al. [3].

A genomic library of *C. pasteurianum* was pre-

Table 1
Specific activities of the enzymes responsible for glycerol fermentation in *C. pasteurianum*[a]

Organism	Specific activity (μmol min^{-1} mg $protein^{-1}$)			
	Glycerol dehydrogenase	Dihydroxyacetone kinase	Glycerol dehydratase	1,3-Propanediol dehydrogenase
C. pasteurianum	4.5	0.10	2.2	1.7
C. freundii	4.3	0.09	1.1	0.9
E. coli ECL707/pRD1	5.4	0.12	1.5	0.8
E. coli ECL707	<0.01	<0.01	-[b]	<0.1
E. coli ECL707/pFL1	<0.01	<0.01	1.4	1.2

[a]Cultures were grown at 30°C and cell extracts were prepared as described in Section 2.
[b]No detectable enzyme activity.

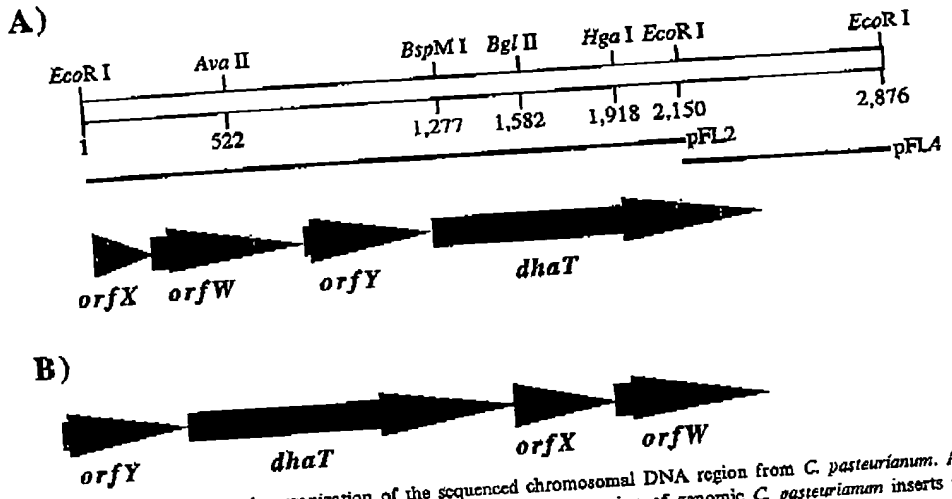
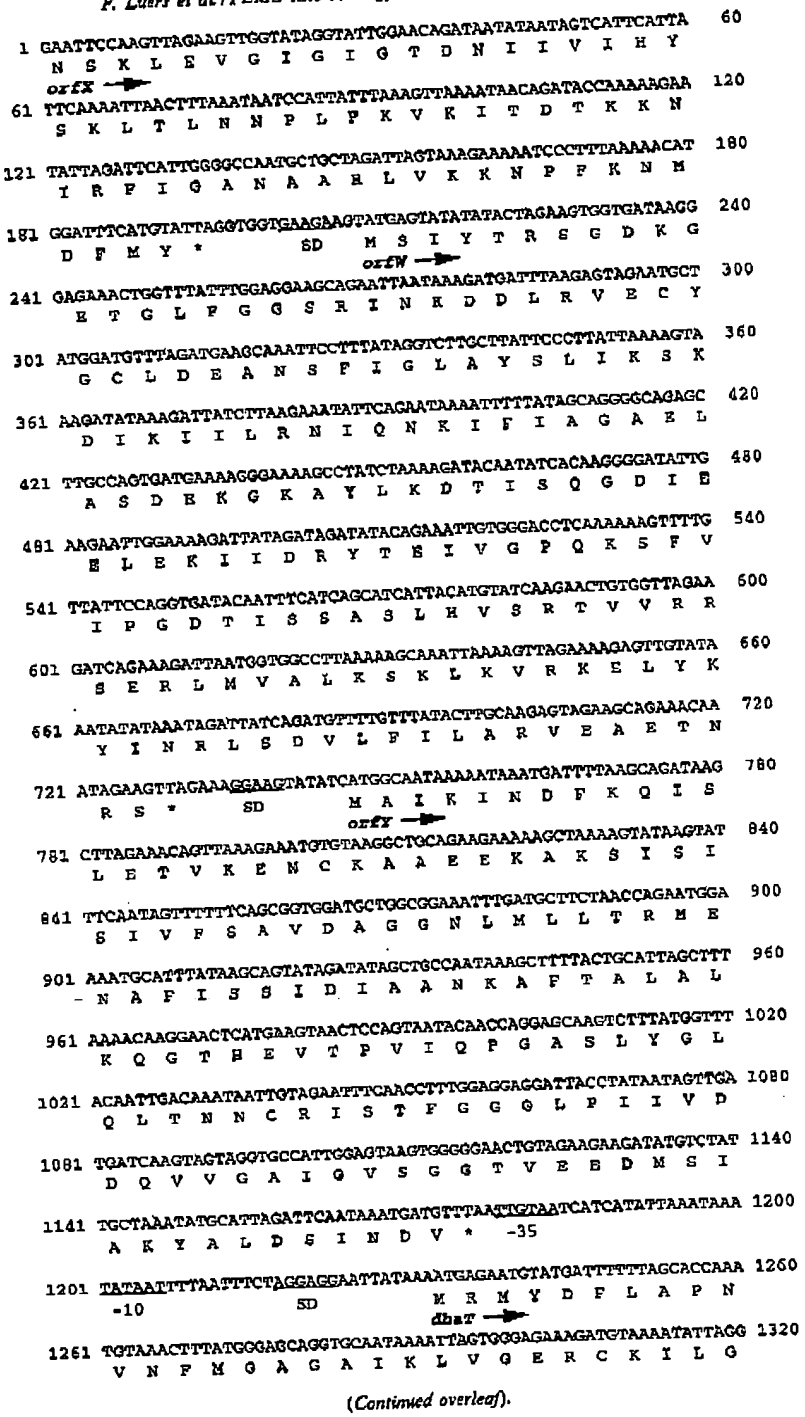


Fig. 2. A: Restriction map and genetic organization of the sequenced chromosomal DNA region from *C. pasteurianum*. Arrows and arrowheads represent length, location and orientation of potential genes. The location of genomic *C. pasteurianum* inserts in recombinant plasmids used for sequencing is given below the restriction map. B: Genetic organization of the homologous DNA region from *C. freundii*.

pared for cloning of the genes involved in glycerol breakdown. Chromosomal DNA was partially digested with *Eco*RI or *Hin*dIII, and ligated into the cosmid pWE15, which had been linearized with the corresponding enzymes. Ligated DNA was packed in vitro into the bacteriophage λ and transduced into the glycerol minus mutant, *E. coli* ECL707. Approximately 2800 recombinant *E. coli* strains with an average insert size of 15 kb were screened on MacConkey-glycerol-ampicillin agar for glycerol utilization. None of these clones had the ability to consume glycerol. This was surprising because this method had been successfully applied for cloning of the entire *dha* regulon from *C. freundii* [5]. Alternatively, the identification of the desired clones in the genomic library was done by colony hybridization using the *dhaT* gene of *C. freundii* as a heterologous DNA probe (data not shown). In this way one clone (*E. coli* ECL707/pFL1) exhibiting glycerol dehydratase and 1,3-propanediol dehydrogenase activity was obtained. The recorded specific activities of 1.4 and 1.2 U mg^{-1}, respectively, were slightly lower than in *C. pasteurianum*, but exceeded those of *C. freundii* (Table 1). Separation of crude extracts by gradient gel polyacrylamide electrophoresis under nondenaturing conditions and activity staining of 1,3-propanediol dehydrogenase gave a single band, corresponding to a native molecular mass of 440 000 Da (Fig. 1). The 1,3-propanediol dehydrogenase produced in *E. coli* ECL707/pFL1 was indistinguishable from the *C. pasteurianum* enzyme with respect to the molecular mass (Fig. 1, lanes 3 and 4). Thus, the genes encoding the reductive branch of glycerol fermentation by *C. pasteurianum* were cloned in *E. coli* ECL707/pFL1. The recombinant cosmid recovered from this strain was designated pFL1 and contained a 13.5-kb

Fig. 3. Nucleotide sequence of the cloned region. Only one strand is shown. The gene encoding 1,3-propanediol dehydrogenase (*dhaT*) and the putative genes *orfW*, *orfX* and *orfY* have been translated using the one-letter amino acid code; amino acid symbols are written below the first nucleotide of the corresponding codon. Potential ribosome binding sites (SD) and putative σ^{70}-dependent promoters are underlined. The putative secondary structure is marked by open arrows indicating the length and orientation of the stem. The sequence has been submitted in full length to GenBank under accession number AF006034.



(Continued overleaf).

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1321 CGGAAAGAAAGCTTTAATAGTTACAGATAAATTCTTGAGAAATATGGAAGATGGAGCTGT 1380
      G K K A L I V T D K F L R N M E D G A V

1381 AGCTCAAACGGTTAAATATATTAAAGAAGCAGGAATAGATGTTGCTTTTTATGATGATGT 1440
      A Q T V K Y I K E A G I D V A F Y D D V

1441 AGAGCCTAATCCTAAGGATACTAATGTTAGAGATGGATTAAAAGTATATAGAAAAGAAAA 1500
      E P N P K D T N V R D G L K V Y R K E N

1501 CTGTGATTTAATAGTTACTGTAGGAGGAGGAAGTTCTCATGACTGTGGAAAGGGAATAGG 1560
      C D L I V T V G G G S S H D C G K G I G

1561 TATTGCAGCTACACACGAGGGAGATCTTTATGACTATGCTGGTATAGAAACCCTTACTAA 1620
      I A A T H E G D L Y D Y A G I E T L T N

1621 TCCATTGCCTCCGATAGTAGCTGTAAATACAACAGCTGGAACAGGCAGCGAGGTTACTCG 1680
      P L P P I V A V N T T A G T G S E V T R

1681 TCATTGTGTTATCACAAACACAAAAACAAAGATTAAATTTGTTATTGTAAGCTGGAGAAA 1740
      H C V I T N T K T K I K F V I V S W R N

1741 TCTGCCGTTAGTATCCATTAATGATCCAATACTTATGATTAAAAAGCCTGCAGGATTAAC 1800
      L P L V S I N D P I L M I K K P A G L T

1801 AGCAGCTACAGGAATGGATGCCTTAACTCATGCCATAGAGTCCTATGTTTCTAAAGATGC 1860
      A A T G M D A L T H A I E S Y V S K D A

1861 AAACCCAGTAACAGATGCCTTAGCTATACAAGCAATAAAATTAATAGCTAACAATCTGCG 1920
      N P V T D A L A I Q A I K L I A N N L R

1921 TCAGGCAGTAGCCCTTGGAGAAAATCTGGAAGCTAGAGAAAATATGGCCTACGCATCACT 1980
      Q A V A L G E N L E A R E N M A Y A S L

1981 TCTGGCAGGAATGGCATTTAATAATGCAAATTTAGGATATGTACATGCTATGGCACATCA 2040
      L A G M A F N N A N L G Y V H A M A H Q

2041 ATTAGGAGGCCTGTATGATATGGCACATGGTGTTGCCAATGCCATGTTATTACCACATGT 2100
      L G G L Y D M A H G V A N A M L L P H V

2101 AGAACGCTATAATCTTATATCAAATCCTAAGAAATTTGCAGATATAGCAGAATTCATGGG 2160
      E R Y N L I S N P K K F A D I A E F M G

2161 AGAGAATATTGAAGGACTTTCAGTAATGGAAGCAGCAGAAAAAGCTATAGATGCTATGTT 2220
      E N I E G L S V M E A A E K A I D A M F

2221 TAGACTTTCAAAGGATGTTGGGATACCAGCAAGTCTTAAAGAAATGGGAGTTAATGAAGG 2280
      R L S K D V G I P A S L K E M G V N E G

2281 AGATTTTGAATATATGGCAAAAATGGCATTGAAAGATGGAAATGCATTCAGTAATCCAAG 2340
      D F E Y M A K M A L K D G N A F S N P R

2341 AAAAGGTAATGAAAAAGATATAGTTAAAATATTTAGAGAAGCATTTTAATTGTAATCTTT 2400
      K G N E K D I V K I F R E A F *
                              >>>>>>>>>>>>>> >>>>   <<<< <<<<<<<<

2401 CATATAATAAGTATTCCTTAGAAATATAAATATATCCTCGATAAGTTTATCAAGGATATA 2460
     <<<<<

2461 TTTATTAAACATTTATAAAAAGCATGAAGGAAGAAGTACATAGAACAATTTATAAGTGA 2520
2521 AATTTTAGAGGGTTACATTTCAATACTGCAGTAGCACCTATTTGTGGAGATGCTGTAGGG 2580
2581 GTATTATTGTATAAAGTTATATTTTAATACCATAATGTGTTGTATGGATTTTAATTAATC 2640
2641 TTATGTATGATTAAAAATATAGTAGAAATATTATGTTTAAATACCTATATAGGAGATCCC 2700
2701 TGATGTATAAAATATTATTAGTTGATGACGAAAATTTAGAAAGAGAAGCCTTGAAAATTA 2760
2761 TGTTAAAAAAACATTAAAGATGAAATAGAAGTTGTAGCCGAAGCTGAAAACGGCAGAGAA 2820
2821 GCTATAGAGCTTGATGAAAAATTAGATCCCTTTAGTGAGGGTTAATTGGGGCCGCGAATT 2880
2881 C
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Fig. 3 (*Continued*).

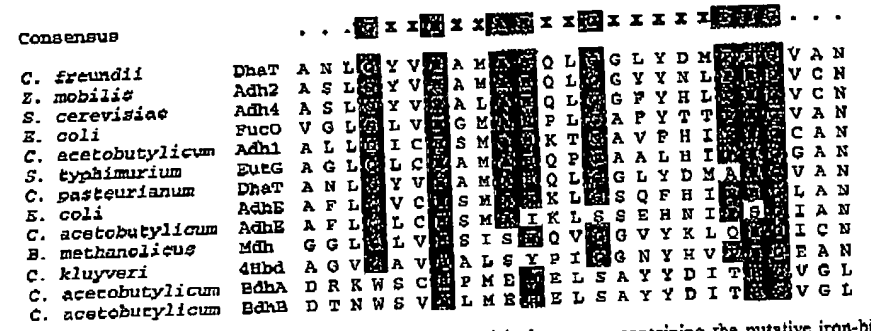

Fig. 4. Amino acid alignment of the protein regions of different alcohol dehydrogenases containing the putative iron-binding motif proposed as a typical feature of class III alcohol dehydrogenases. Shaded amino acids indicate the core motif postulated by Bairoch [21]. The consensus of the putative iron-binding motif was compared with 1,3-propanediol dehydrogenase (DhaT) of *C. freundii* [7], alcohol dehydrogenase (Adh2) of *Z. mobilis* [28], alcohol dehydrogenase (Adh4) of *S. cerevisiae* [26], 1,2-propanediol dehydrogenase (FucO) of *E. coli* [23], alcohol dehydrogenase (Adh1) of *C. acetobutylicum* [29], ethanolamine utilization protein (EutG) of *S. typhimurium* [25], 1,3-propanediol dehydrogenase of *C. pasteurianum* (DhaT), 4-hydroxybutyrate dehydrogenase (4Hbd) of *C. kluyveri* [30], alcohol dehydrogenase (AdhE) of *E. coli* and *C. acetobutylicum* [31,32], methanol dehydrogenase (Mdh) of *Bacillus methanolicus* C1 [33], and the two butanol dehydrogenase isoenzymes (BdhB and BdhA) of *C. acetobutylicum* [34].

insert of *C. pasteurianum* genomic DNA. To subclone the *dhaT* gene encoding 1,3-propanediol dehydrogenase, pFL1 was digested with *Eco*RI and the fragments were ligated into pBluescript SK+. Colony hybridization with the DNA probe from *C. freundii* (see above) revealed that the complete *dhaT* gene of *C. pasteurianum* was located on two recombinant *E. coli* strains with different inserts, one containing a 2155-bp and the other a 732-bp *Eco*RI fragment of genomic *C. pasteurianum* DNA. The plasmids isolated from these strains were designated pFL2 and pFL4, respectively. The origin and the neighborhood on the chromosome of both cloned *Eco*RI fragments was established by Southern blot analysis (data not shown).

The inserts of pFL2 and pFL4 were sequenced in both directions. The restriction map and the apparent gene organization are shown in Fig. 2A, and the sequence of the combined *Eco*RI fragments from pFL2 and pFL4 (2881 bp) is given in Fig. 3. Four successive potential genes were identified within the sequence. One gene is located at the end of the cloned DNA and is hence incomplete. All presumptive genes except the incomplete one were preceded by a potential ribosome-binding site, appropriately spaced from the start codon (Fig. 3). The deduced amino acid sequences of the four open reading frames showed high similarity to OrfW, OrfX, OrfY and DhaT, which are part of the *dha* regulon of *C. freundii* [6–8]. The *C. pasteurianum* genes were designated accordingly.

The *dhaT* gene (1158 bp) of *C. pasteurianum* encodes 385 amino acids with a predicted molecular mass of 41 776 Da. The *dhaT* gene is terminated by a single stop codon (UAA). A sequence that could represent a transcriptional terminator (a punctated palindrome that could form a stem-loop structure in an RNA transcript) follows approximately 36 nucleotides downstream from the stop codon (Fig. 3). A conserved sequence for σ^{70}-dependent promoters is located upstream of the *dhaT* gene in positions 1178–1206 (Fig. 3).

The amino acid sequence deduced from *dhaT* of *C. pasteurianum* was compared with deduced amino acid sequences from alcohol dehydrogenases available in the NCBI databases. The highest similarity (80.5% identity and 89.8% similarity) was obtained to the 1,3-propanediol dehydrogenase of *C. freundii*, which is a member of a novel family of alcohol dehydrogenases (type III). This high amino acid sequence identity corresponded well with the similar native molecular mass of both enzymes observed during nondenaturing electrophoresis (Fig. 1). The 1,3-propanediol dehydrogenase of *C. freundii* is a decamer of a polypeptide of 43 400 Da under these conditions [19]. The predicted molecular mass of the

dhaT gene product (41776 Da) and the estimated native molecular mass (440000 Da) suggest the same subunit composition for 1,3-propanediol dehydrogenase of *C. pasteurianum*.

The family of type III alcohol dehydrogenases is very heterogeneous and distinct from the long-chain zinc-containing (type I) or short-chain zinc-lacking (type II) enzymes [20]. The other members of type III alcohol dehydrogenases, including e.g. Adh2 of *Zymomonas mobilis* and FucO of *E. coli* (for other enzymes, see Fig. 4), exhibited 28.3–51.6% identity (51.1–70.8% similarity) to 1,3-propanediol dehydrogenase from *C. pasteurianum*. No significant similarities between 1,3-propanediol dehydrogenase and type I and type II alcohol dehydrogenases were found.

Bairoch [21] proposed a more or less conserved putative iron-binding motif (G-X-X-H-X-X-A-H-X-X-G-X-X-X-X-X-P-H-G) as a fingerprint pattern for type III alcohol dehydrogenases (Fig. 4). It is fully conserved in all reported iron-dependent enzymes (DhaT from *C. freundii* [7], Adh2 from *Z. mobilis* [22], FucO and AdhE from *E. coli* [23,24]), in EutG from *Salmonella typhimurium* with unknown ion requirement [25] and in Adh4 from *Saccharomyces cerevisiae*, which requires Zn^{2+} for its catalytic activity [26]. The *dhaT* gene product showed the iron-binding motif (amino acids 262–280), except that the conserved proline in position 278 was replaced by alanine (Fig. 4). The ion requirement of the enzyme has not been determined but iron limitation during growth on glycerol favors the formation of 1,3-propanediol and reduces the production of the other solvents butanol and ethanol [3]. This makes an iron-dependent 1,3-propanediol dehydrogenase unlikely.

1,3-Propanediol dehydrogenase requires NAD(H) as a cofactor, but the highly conserved NAD(H) binding fingerprint pattern G-X-G-X-X-G [27] was not present in the amino acid sequence. This is also characteristic of most type III alcohol dehydrogenases.

The deduced products of the remaining three presumptive genes, *orfY*, *orfW* and the incomplete *orfX*, exhibited 31.5–53.8% identity (45.9–69.9% similarity) to the corresponding homologous gene products encoded by the *dha* regulon of *C. freundii*. In comparison to this organism the sequenced genes of *C. pasteurianum* showed a different organization; *orfX*, *orfW*, *orfY* were all located upstream of the *dhaT* gene (Fig. 2).

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